ANNEX D

JOINT MEDICAL CHEMICAL, BIOLOGICAL, AND NUCLEAR DEFENSE RESEARCH PROGRAMS

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JOINT MEDICAL CHEMICAL, BIOLOGICAL, AND NUCLEAR DEFENSE RESEARCH PROGRAMS

The joint medical chemical, biological, and nuclear (radiological) defense research programs are each addressed in the next three sections.

D.1 MEDICAL CHEMICAL DEFENSE RESEARCH & ACQUISITION PROGRAM

D.1.1 Fielded Products

Advances in medical research and development (R&D) significantly improve the warfighting mission by sustaining unit effectiveness through conserving the fighting strength of our forces and supporting the nation's global military strategy, which requires the ability to effectively deploy and operate. Medical R&D products (materiel and non-materiel solutions) provide the foundation that ensures the fielding of a flexible, sustainable, modernized force across the spectrum of conflict and in the full breadth and depth of the battlefield. Overcoming medical threats and extending human performance has provided a significant increase in military effectiveness in the past and presents the potential for future enhancement of military operational effectiveness. Some fielded materiel and non-materiel solutions by medical chemical defense R&D are:

Pharmaceuticals (See Figure D-1):

- Nerve Agent Antidote Kit (Mark I), 1983
- Skin Decontamination Kit (M291), 1990
- Nerve Agent Pretreatment (Pyridostigmine), 1991*
- Convulsant Antidote for Nerve Agent (CANA), 1991*
- Medical Aerosolized Nerve Agent Antidote (MANAA), 1993*

* Initial fielding of these medical products was funded under Low Rate Initial Production (LRIP) options in developmental contracts with RDT&E dollars. Therefore, the following FY96 actions were accomplished: (1) Proved long term extended stability of the medical aerosolized nerve agent antidote (MANAA), the convulsant antidote for nerve agent (CANA), and the nerve agent pretreatment (pyridostigmine), (2) Completed one year follow-up to pyridostigmine gender study, and (3)†submitted new drug application (NDA) for pyridostigmine to the FDA.

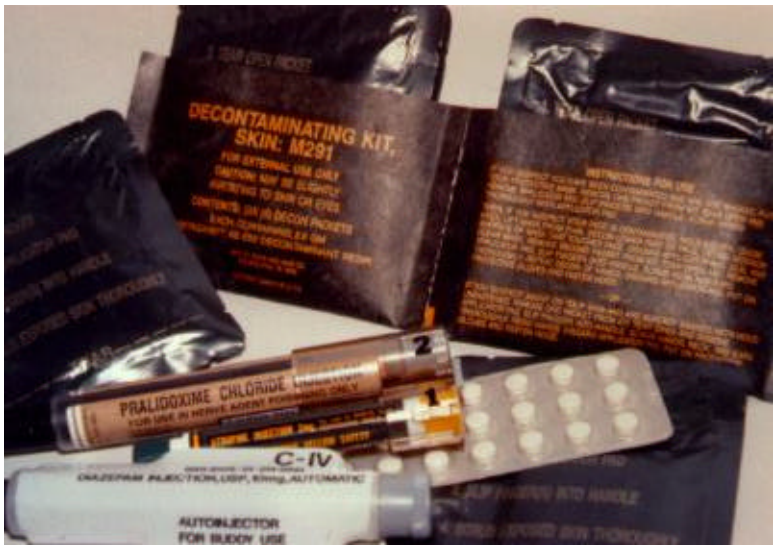


Figure D-1. Field Pharmaceutical Products

Materiel (See Figure D-2):

- Resuscitation Device, Individual, Chemical, 1990
- Decontaminable Patient Litter (NSN 6530-01-380-7309), 1991
- Chemical Warfare (CW) Protective Patient Wrap (NSN 8415-01-311-7711), 1991
- Computer-Based Performance Assessment Battery, 1993
- M40 Protective Mask Vision Correction (optical inserts)

Figure D-2. Decontaminable Patient Litter and CW Patient Wrap

Information and Doctrine:

- Taxonomic Work Station, 1985
- U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) Technical Memoranda on Chemical Casualty Care, 1990

- Field Manual (FM) 8-285, “Treatment of Chemical Agent Casualties and Conventional Military Chemical Injuries,” 1990
- Handbook, “Medical Management of Chemical Casualties,” 1995
- Field Management Handbook, “Medical Management of Chemical Casualties,” 1996
- Technical Bulletin (TB) Medical (MED) 296, 1996
- Compact Disk - Read-Only Memory (CD-ROM) on “Management of Chemical Warfare Injuries,” 1996
- Presented instruction on the medical management of chemical casualties (19 courses that trained 1,797 personnel in 1996 including over 600 civilian “first responders”), as shown in Figure D-3.

Figure D-3. Medical Management of Chemical Casualties

D.1.2 Medical Chemical Defense Research and Development Accomplishments

The medical chemical defense research and development technical barriers and accomplishments during FY96 are grouped by the classical chemical threat categories, which include the following:

- Vesicants or blister agents (*e.g.*, sulfur mustard [HD] and Lewisite),
- Nerve agents (*e.g.*, soman [GD], VX),
- Blood agents (*e.g.*, cyanide), and
- Respiratory agents (*e.g.*, phosgene).

The chemical threat, however, is not restricted to commonly accepted classical agents. Novel agents may be developed by potential adversaries. The ability to provide timely and effective medical countermeasures to new threats depends upon maintaining a high level of technological capability.

Countermeasures to these threats include pharmaceuticals, medical equipment, specialized materiel or medical procedures, and concepts for training, doctrine, and organization. Medical countermeasures are designed not only to prevent lethality but to preserve and sustain combat effectiveness in the face of combined threats from chemical and conventional munitions on the integrated battlefield by:

- Prevention of the effects of chemical agents (*e.g.*, pretreatments or prophylaxes),
- Far-forward treatment upon exposure to chemical warfare threats (*e.g.*, antidotes),
- Chemical casualty care (*e.g.*, therapy and management).

THREAT CATEGORY: VESICANT AGENTS

The countermeasures, technical barriers, and accomplishments in the chemical threat category of vesicant agents are outlined below.

Countermeasures:

- Topical protectants to protect skin against blister agents
- Biological/pharmaceutical products to prevent cell death caused by vesicant agents

Technical Barriers:

- Appropriate experimental model systems to predict drug or treatment efficacy and safety in humans
- Pretreatments/antidotes with special characteristics, such as quick action, long-lasting, easy to carry and use
- Reactive/catalytic decontaminant activity versus safety of decontaminant and protectant compounds

Accomplishments:

- Defined and published six medical countermeasure strategies against vesicant agents: intracellular scavengers, cell cycle inhibitors, poly-adenosine diphosphate (ADP) ribose polymerase inhibitors, calcium modulators, protease inhibitors, and anti-inflammatory agents.

- Demonstrated that HD can inhibit a mitochondrial metabolic pathway.
- Demonstrated increased expression of antigens related to a modified differentiation state of keratinocytes following HD exposure.
- Showed that activation of deoxyribonucleic acid (DNA) ligase I in HD exposed keratinocytes involves phosphorylation.
- Determined that fragment crystallizable (Fc) receptor activity, a critical step in generation of an inflammatory reaction, is upregulated in HD exposed keratinocytes.
- Established that oxidation of thiodiglycol is necessary for generation of protein phosphatase inhibition and that this can occur in human skin.
- Developed magnetic biotechnological techniques (electron paramagnetic resonance and nuclear magnetic resonance) for quantitation of reactive species and an enzyme-linked immunosorbent assay (ELISA) for cytokine expression following exposure of cells and tissues to HD.
- Determined that a human organotypic skin model for HD exposure expresses all the ultrastructural components of normal human skin.
- Used human skin biopsies for histochemical and immunohistochemical studies of the pathogenesis of the HD lesion.
- Determined that liquid exposure to HD in the mouse ear model resulted in reproducible edema and subepidermal blister formation.
- Determined that 15 minute cutaneous vapor HD exposure of the weanling pig model reproducibly results in erythema and microblister formation.
- Demonstrated that ethacrynic acid, an inhibitor of glutathione transferase, could elevate intracellular glutathione levels to 200-300% of control levels.
- Demonstrated that calcium chelators and phospholipase A2 inhibitors could block the HD induced release of arachidonic acid from normal human keratinocytes.
- Screened numerous compounds for their ability to prevent HD-initiated loss of adenosine triphosphate (ATP). Four compounds were found to be significantly more effective than niacinamide.
- Identified candidate antiproteases with 100% inhibition of HD-increased protease activity.
- Refined and validated *in vitro* and *in vivo* testing procedures, and developed a database for evaluation of HD injury.
- Began a protocol to study the effects of anti-inflammatory and/or protease inhibitory drugs on increased proteolysis and histopathology associated with HD cutaneous injury.
- Synthesized guanine-nitrogen mustard and guanine-sulfur mustard adducts as internal standards for quantification of vesicant activity and antivesicant efficacy.
- Determined that sulfur and nitrogen mustards stimulate protease activity in human skin cells *in vitro* and in the mouse ear model *in vivo*.
- Demonstrated that mustard-stimulated protease is calcium dependent and is likely a serine protease.
- Developed a rabbit eye model for HD eye injury and then evaluated anti-inflammatory agents, antibiotics and protective contact lenses as post-exposure treatments or as prophylaxes to ocular liquid sulfur mustard exposure.
- Refined technique to evaluate laser debridement of HD burns.

- Determined that laser wound debridement was an effective accelerator of wound healing in skin exposed to sulfur mustard vapor.
- Evaluated temporary wound dressings in conjunction with surgical debridement of skin exposed to sulfur mustard vapor.
- Developed effective methods to detect 2-chlorovinylarsenous acid (CVAA) in the urine of guinea pigs exposed to Lewisite.

THREAT CATEGORY: NERVE AGENTS

The countermeasures, technical barriers, and accomplishments in the chemical threat category of nerve agents are outlined below.

Countermeasures:

- Topical protectants to protect skin against thickened nerve agents
- Pretreatment regimen that protects against incapacitating effects
- Improved antidote to treat incapacitating effects
- Anticonvulsant antidote to prevent or minimize convulsions and brain injury

Technical Barriers:

- Appropriate experimental model systems to predict drug or treatment efficacy and safety in humans
- Pretreatment/antidotes with special characteristics, such as quick action, long-lasting, easy to carry and use
- Central nervous system (CNS) active drugs with acceptable side-effects
- Generation of immune response to small molecules

Accomplishments:

- Determined that potent centrally active anticholinergics are the class of drugs to recommend for Milestone 0 transition as advanced anticonvulsants; identified four leading compounds for further testing.
- Developed a double mutant of human butylcholinesterase (hBuChE) that ages slowly and catalyzes the hydrolysis of soman and other nerve agents to be used as a second generation antidote for nerve agents.
- Submitted portable field blood cholinesterase test system to the Food and Drug Administration (FDA) in FY 96.
- Developed effective methods to detect the specific degradation products of nerve agents (VX and tabun (GA)) in spiked urine samples.
- Initiated new method to facilitate isolation of hBuChE and its mutants from cell culture medium.
- Established and validated 2 novel expression systems for hBuChE to speed production of mutants for testing.

- Conducted research which showed that rhesus monkeys trained under a Serial Probe Recognition (SPR) task showed no behavioral deficits when administered 25,000 units of unpurified BuChE, sufficient enzyme to protect against two median lethal doses of soman.
- Covalently coupled highly purified acetylcholinesterase to a synthetic sponge for development of a nerve agent decontaminating sponge when soaked in an oxime solution.
- Developed cellular expression system for human carboxylesterase (hCaE).
- Altered C-terminal amino acid of hCaE to allow for secretion of the bioscavenger into the medium for ease of production and purification.
- Produced three different antibody fragments (fragment antigen binding (Fabs)) for use in immunoassays.
- Tested five monoclonal antibodies for binding against soman acid analogues. The Fabs are being purified for competitive binding assays against soman acid.
- Identified a monoclonal anti-soman antibody useful for diagnosing, in an ELISA, a sublethal soman exposure.
- Developed model of seizures and brain damage; time-dependent sequential activation of cholinergic and glutamatergic receptors.
- Evaluated anticonvulsant effectiveness of experimental drugs, antiepileptic drugs, and neuroprotectants.
- Determined factors that influence seizure expression with nerve agents (GA, GB, VX).
- Initiated study to determine the utility of using cardiac isoenzymes as biomarkers of cardiac damage following nerve agent-induced seizures.
- Advanced evaluation of anticonvulsant effectiveness of anticholinergics versus diazepam.
- Extended use of cholinesterase-oxime combinations by covalently linking enzyme within polyurethane foam for potential use in skin and wound decontamination.
- Demonstrated that Huperzine-A afforded protection of animals from soman poisoning, reduced neuronal cell death, and reduced soman-induced seizures.

THREAT CATEGORY: BLOOD AGENTS

The countermeasures, technical barriers, and accomplishments in the chemical threat category of blood agents are outlined below.

Countermeasures:

- Pretreatment compounds to protect against rapid action of these chemical agents

Technical Barriers:

- Appropriate experimental model systems to predict drug or treatment efficacy and safety in humans
- Pretreatments/antidotes with special characteristics, such as quick action, long-lasting, easy to carry and use

Accomplishments:

- Synthesized potential metabolites of cyanide antidotes.
- Resolved racemic cyanide antidote into pure optical isomers.
- Concluded animal toxicology studies for cyanide pretreatment.
- Demonstrated extended stability of the cyanide pretreatment product.

THREAT CATEGORY: RESPIRATORY AGENTS

The countermeasures, technical barriers, and accomplishments in the chemical threat category of respiratory agents are outlined below.

Countermeasures:

- Short-term: Health risk criteria for emerging threat doctrine, care and treatment strategies
- Intermediate-term: Specific casualty management techniques to improve survival and minimize lost duty time
- Long-term: Pharmaceutical/biological pretreatments, antidotes, or decontaminants/ protectants

Technical Barriers:

- Appropriate experimental model systems to predict drug or treatment efficacy and safety in humans
- Pretreatment/antidotes with special characteristics, such as quick action, long-lasting, and easy to carry

Accomplishments:

- Developed the miniature swine inhalation model to evaluate common clinical endpoints utilized in human intensive care, including clinical chemistry and hematology, x-rays, pulse oximetry, impedance plethysmography and arterial blood gas analysis.
- Demonstrated significant efficacy of ibuprofen and verapamil as treatment in mice exposed to phosgene.

D.1.3 Advanced Development Products

In advanced development, the goal is proof-of-principle. Efforts in this category are directed toward the solution of identified deficiencies. The medical R&D process links the materiel developer (U.S. Army Medical Research and Materiel Command (USAMRMC)) with the combat and training developer (Army Medical Department Center and School (AMEDDC&S)) and the logistician in addressing the threat and Department of Defense (DoD) requirements. Medical chemical defense products now in the advanced development phase are

the following:

PRODUCT: TOPICAL SKIN PROTECTANT (TSP)

Concept:

- Use perfluorinated formulations.
- Form non-toxic, non-irritating barrier film layer on skin.
- Augments Mission Oriented Protective Posture (MOPP)
- Protection against vesicant and nerve agents

Status:

- Two candidates transitioned to demonstration-validation phase
- Candidates demonstrated efficacy against broad spectrum of threat agents
- Investigational New Drug (IND) application submitted to the FDA
- Demonstrated the human safety and technical performance of the topical skin protectant
- Demonstrated extended stability of the topical skin protectant
- Validated production/manufacturing capability for the topical skin protectant.
- Began research for a safe and efficacious reactive component for a second generation reactive topical skin protectant (rTSP) that will provide equivalent protection against penetration and will detoxify both vesicant and nerve chemical warfare agents. Toward this goal, established a working list of candidate reactive moieties for a rTSP and for wound decontamination systems coupled to a research plan to acquire and evaluate them.

PRODUCT: MULTICHAMBERED AUTOINJECTOR

Concept:

- Speed administration of life-saving antidotes against nerve agents
- Replace 2 Injector Mark I Nerve Agent Antidote Kit with single autoinjector

Status:

- Engineering contract awarded in September 1993
- Fielding will require full FDA approval
- Demonstrated the human safety and technical performance of the multi-chambered autoinjector
- Demonstrated extended stability of the multi-chambered autoinjector

PRODUCT: CYANIDE PRETREATMENT

Concept:

- Provide protection against incapacitation and lethality without performance degradation
- Enhance soldier protection and sustainment

Status:

- Lead component transitioned to advanced development
- Completed pre-clinical toxicology and drug distribution studies
- Developed dose parameters and performance assessments
- Concluded animal toxicology studies for cyanide pretreatment
- Demonstrated extended stability of the cyanide pretreatment product

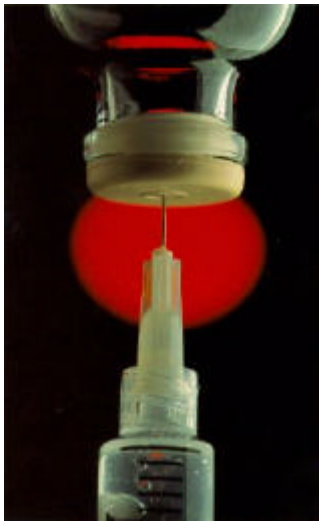
D.2 MEDICAL BIOLOGICAL DEFENSE RESEARCH & ACQUISITION PROGRAM

D.2.1 Biological Defense Products

Advances in DoD medical R&D significantly impact the warfighting mission by sustaining unit effectiveness through conserving the fighting strength of our soldiers and supporting the nation's global military strategy which requires the ability to effectively deploy and operate. Medical R&D products (materiel and non-materiel solutions) provide the foundation that ensures the fielding of a flexible, sustainable, modernized force across the spectrum of conflict and in the full breadth and depth of the battlefield. Overcoming medical threats and extending human performance has provided a significant increase in military effectiveness in the past and presents the potential for future enhancement on military operational effectiveness. Some of the materiel and non-materiel solutions by medical biological defense R&D include the following:

Vaccines:

- Anthrax Vaccine (licensed)
- Botulinum Toxoid Vaccine, Pentavalent (IND #3723)
- Botulinum Type F Toxoid Vaccine (IND #5077)
- Botulism Immune Globulin (F(ab')2), Equine (IND #3703)
- Botulism Immune Globulin, Human (IND #1332)
- Eastern Equine Encephalitis Virus Vaccine (IND #266)
- Q Fever Vaccine, Purified Whole Cell, CM Residue, Formalin Inactivated, Gamma Irradiated (IND #3516)
- Tularemia Vaccine (IND #157)
- Vaccinia Virus Vaccine, Cell Cultured (IND #4984)
- Venezuelan Equine Encephalomyelitis Virus Vaccine, TC-83 (IND #142)
- Western Equine Encephalitis Virus Vaccine (IND #2013)



Information and Doctrine:

- Handbook "Medical Management of Biological Casualties," 1996
- Presented instruction on the medical management of biological casualties (19 courses that trained 1,797 personnel in 1996 including over 600 civilian "first responders")

D.2.2 Biological Defense Research and Development Accomplishments

The biological defense research and development technical barriers and accomplishments during FY96 are grouped by biological threat category, which include the following:

- Bacterial (and rickettsial) agents,
- Protein toxins, and
- Viral agents

In addition, research and development accomplishments in the area of confirmatory assays for biological warfare threat agents is presented at the end. The objective of this effort is to develop the capability to confirm in biological samples the initial field diagnosis of a biological warfare threat agent.

THREAT CATEGORY: BACTERIAL AGENTS

The countermeasures, technical barriers, and accomplishments in the biological threat category of bacterial agents are outlined below.

Countermeasures:

- Vaccines for immunity against threat agents
- Antibiotics for treatment of bacterial diseases
- Forward deployed diagnostic systems

Technical Barriers:

- Incomplete genetic information for all the threat agents
- Appropriate animal model systems for investigation of bacterial threats and countermeasures
- Capability to produce Good Manufacturing Practice (GMP) pilot lots of vaccine candidates
- Inability to perform human clinical trials to prove efficacy of vaccines
- Difficulty in optimizing and comparing different expression vectors for recombinant products
- Immunogenicity of vaccine
- Difficulty in field testing rapid identification kits under natural conditions
- Defining surrogate markers of protection

Accomplishments:

Anthrax

- Demonstrated in the rabbit model of inhalation anthrax that two doses of 5 or 0.5 μg of recombinant anthrax protective antigen (PA) are protective.

- Completed comparison of efficacy of the recombinant PA with the current licensed vaccine in the non-human primate model of inhalation anthrax and demonstrated that two doses of 5 or 0.5 µg of recombinant PA is protective. This protection is comparable to that achieved by the licensed human vaccine.
- Completed comparison of efficacy of recombinant PA combined with different adjuvants in the non-human primate model of inhalation anthrax.
- Developed antitoxin neutralization assays.
- Demonstrated partial passive protection of rabbits from subcutaneous and aerosol challenge with hyperimmune serum.
- Presented research plan to the Joint Program Office for Biological Defense and the FDA in pre-IND meeting concerning a proposed amendment of the anthrax vaccine adsorbed license to reduce the number of required doses and to include an indication for aerosol exposure.
- Approved protocols and qualified a contracting facility for producing a master cell bank of the non-sporulating delta Sterne-1 (pPA102) CR4 anthrax strain for production of recombinant PA.
- Prepared IND to evaluate optimal two doses regimen of licensed human anthrax vaccine in humans.

Plague

- Demonstrated that aminoglycoside alternatives to streptomycin and fluoroquinolones are effective in post-exposure treatment of experimental plague pneumonia while beta-lactam antibiotics are ineffective if given late after exposure.
- Demonstrated efficacy of recombinant Fraction 1 (F1) capsule in protecting against experimental pneumonic plague in rodents.
- Demonstrated efficacy of recombinant V antigen in protecting against experimental pneumonic plague caused by F1-positive or F1-negative strains of *Yersinia pestis*.
- Demonstrated efficacy of recombinant Fraction 1 - "V" antigen (F1-V) fusion protein in protecting against experimental pneumonic plague in rodents caused by F1-positive or F1-negative strains.
- Submitted patent application for use of recombinant F1-V antigen fusion protein as candidate plague vaccine.
- Cloned, expressed, purified, and tested nine additional virulence factors as potential vaccine candidates, showing that only one showed any efficacy against experimental infection.
- Demonstrated that siderophore-mediated iron uptake but not hemin utilization is necessary for virulence of *Yersinia pestis* from a subcutaneous route.
- Developed *in vivo* and *in vitro* models to determine mechanism of action and immunity to V antigen.
- Developed ELISA and Western Blot assays to comprehensively analyze the immune response of rodents to plague infection.

Glanders

- Acquired ten strains of *Burkholderia mallei*, the causative agent of glanders, and

completed *in vitro* growth conditions and biochemical characterization.

- Determined decontamination procedures and irradiation kill curves for *Burkholderia mallei.*
- Evaluated seventeen antibiotics *in vitro* against a single virulent strain.
- Completed initial median lethal dose (LD_{50}) determinations for two strains in hamsters.
- Generated rabbit anti-whole cell sera as initial diagnostic reagents.

Brucellosis

- Developed double deletion mutants of a *Brucella* species.
- Demonstrated efficacy of two vaccine candidates against intranasal challenge in mice.

Typhus

- Characterized human and mouse humoral and cellular immune responses to typhus infection.
- Developed mouse and guinea pig protection models.
- Demonstrated surface protein antigen (SPA) subunit vaccine efficacy in eliciting protection against typhus in mice and guinea pigs.
- Mapped human T-cell epitopes on SPA.
- Chemically characterized SPA and cloned SPA gene.
- Identified phage expression epitopes for toxin neutralizing antibodies against *Rickettsia prowazekii*.
- Partially characterized methylation sites on SPA.
- Studied proteolytic processing of SPA precursor to mature surface antigen used as vaccine.
- Designed primers for cloning and overexpressing segments of SPA.

THREAT CATEGORY: PROTEIN TOXINS

The countermeasures, technical barriers, and accomplishments in the biological threat category of protein toxins are outlined below.

Countermeasures:

- Antibodies (antitoxins) directed against common antigens of protein toxin molecules
- Vaccines for immunity against protein toxin threat agents
- Confirmatory assays to identify protein toxins specifically or classes of protein toxins
- Drugs for supportive therapy of agent intoxication

Technical Barriers:

- Capability to produce GMP pilot lots of vaccine candidates
- Inability to perform human clinical trials to prove efficacy of vaccines and antitoxins
- Difficulty in optimizing and comparing different expression vectors for recombinant products

- Immunogenicity of vaccine and vaccine delivery technology
- Difficulty in field testing diagnostic kits under natural conditions
- Difficult to produce polyvalent vaccines against toxin classes
- Lack of rapid confirmatory assays with "gold standard" sensitivity and specificity
- Appropriate animal model systems for investigation of protein toxin threats and countermeasures
- Defining surrogate markers of protection

Accomplishments:

Botulinum Toxin

- Demonstrated a significant delay in the onset of botulinum toxin-induced muscle paralysis by appropriate combinations of a receptor blocker, zinc chelator, metalloprotease inhibitor and aminoquinoline compound.
- Successfully expressed a synaptobrevin peptide by recombinant DNA techniques in an *Escherichia coli* strain. This peptide was rapidly cleaved by botulinum neurotoxin (BoNT) and is currently used in cell-free assays to test potential BoNT inhibitors.
- Optimized capillary electrophoresis conditions for detection of the synaptobrevin peptide. The parameters optimized included voltage, ionic strength, pH, and reactant concentrations (BoNT/B light chain and substrate)
- Designed three dipeptide phosphoramidate compounds that are complimentary to the BoNT/B cleavage site in synaptobrevin. Preliminary cell-free assays indicate that the phenyl analog is able to reduce the cleavage rate of BoNT/B but the methyl and ethyl analogs are relatively inactive.
- Demonstrated that the clinically used antihypertensive agent, Captopril, can inhibit cleavage of the synaptobrevin peptide. Captopril was only marginally effective in protecting isolated muscles from BoNT/B action, presumably due to its poor penetration of plasma membranes.
- Examined the clinically used antimalarial agent, chloroquine, for its ability to reduce the rate of BoNT/A mediated muscle paralysis in the mouse phrenic nerve hemidiaphragm preparations. Chloroquine produced a 3 fold slowing in the time-to-paralysis when added prior to or simultaneously with BoNT/A.
- Performed structure-activity studies with additional aminoquinoline and acridine compounds. The resulting rank order potencies (in decreasing order) were: quinacrine, amodiaquine, chloroquine, and quinine. Primaquine and WR242511 were ineffective.
- Performed molecular modeling studies to determine the salient features of the BoNT-mediated transmembrane channel responsible for translocation of the light chain into the cytosol. The channel is formed by four regions of the N-terminal half of the heavy chain and has a negatively charged lumen.
- Demonstrated that the effective aminoquinoline compounds can block this channel by providing complimentary groups for hydrogen bonding and electrostatic and hydrophobic interactions with the channel interior. The inactive antimalarial compounds (Primaquine and WR242511) lacked these features and were unable to bind effectively to the BoNT channel lumen.

- Established optimal combinations of potential therapeutic agents for antagonizing the binding, translocation and proteolytic activity of BoNT/A and /B in the phrenic nerve hemidiaphragm preparation.
- Synthesized and inserted in intracellular yeast vector all 7 recombinant botulinum toxin fragment C regions for serotypes A, B, C1, D, E, F and G.
- Completed Master Cell Bank and Master Cell Production Bank for botulinum neurotoxin heavy chain (Hc) at Walter Reed Army Institute of Research (WRAIR) GMP pilot lot facility.
- Initiated development of a potency test for recombinant botulinum toxin vaccines.
- Initiated excipient study to determine optimal cryoprotectants and stabilizers for vaccine formulations.
- Initiated aerosol dose-ranging studies for all 7 botulinum toxin serotypes in non-human primates.
- Initiated simultaneous comparative study of in-house botulinum toxin mouse serum neutralization bioassay and two botulinum toxin ELISAs, to assess correlation between the three assays and to explore possibility of replacing bioassay with ELISA.
- Filed invention disclosure which describes botulinum toxin enzymatic cleavage assay and potential uses for it as a research, diagnostic or detection tool.
- Synthesized 17 peptide substrates for botulinum toxin A, four of which inhibit toxin activity, to be characterized further as toxin antagonists.
- Developed affinity-purified polyclonal antibodies to detect botulinum toxin A, B, and E in clinical and field samples.
- Developed and still characterizing neutralizing monoclonal antibodies against botulinum toxin A, E, and F as research tools.
- Synthesized phosphoramidon-like peptides as botulinum antidotes.
- Developed rapid *in vitro* assay for botulinum B serotype.
- Screened eight compounds for inhibition of botulinum endopeptidase activity.
- Developed production plan for and produced GMP lot of botulinum B seed material.
- Discovered that mastoparan, a releaser of arachidonic acid, protects against botulinum toxin A via increased vesicle fusion in PC12 cells *in vitro*.
- Developed sensitive and specific hand-held assay for botulinum toxins A and B.

Staphylococcal Enterotoxin B (SEB)

- Expressed triple mutant SEB vaccine in bacterial and yeast vectors.
- Expressed triple mutant staphylococcal enterotoxin A (SEA) vaccine in bacterial vector.
- Demonstrated that triple mutant SEB vaccine protects non-human primates from lethal aerosol SEB challenge.
- Fully characterized and reported the lethal aerosol SEB non-human primate model.
- Fully characterized and reported the lipopolysaccharide (LPS)-potentiated lethal aerosol SEB rodent model.
- Developed an incapacitating aerosol SEB rodent model.
- Developed ability to detect SEB in picogram quantities in biosamples using specialized mass spectrometry methods.
- Produced affinity purified polyclonal antibodies to detect SEB in biosamples by ELISA.

- Developed surrogate marker assays (ELISA and lymphocyte proliferation inhibition) to predict protective immunity induced by SEB vaccines.
- Identified free-radical scavenger heteropoly anions, 2 anti-cytokine antibodies, 2 cytokine inhibitory drugs, an SEB peptide fragment, and two other FDA approved drugs as viable therapeutic candidates against SEB exposure.
- Developed capability for GMP production of SEB toxin and proteosomes, and methodology for mass production of two mutant protein vaccine candidates.
- Developed sensitive and specific hand-held assay for staphylococcal enterotoxin B.

Ricin

- Produced and bottled a GMP pilot lot of lyophilized, deglycosylated ricin A-chain (DGA) vaccine candidate and initiated characterization and stability testing of this vaccine pilot lot.
- Developed and designed a potency test for the DGA vaccine candidate.
- Developed and characterized a lethal mouse ricin aerosol model to assess immunogenicity and survivability conferred by ricin vaccines.
- Developed and characterized a lethal rat ricin aerosol model to assess immunogenicity, survivability, and protection from lung injury conferred by ricin vaccines.
- Developed ability to detect ricin in nanogram quantities in biosamples using specialized mass spectrometry methods.
- Compared two in-house, established cell culture ricin serum neutralization tests, to select a test for standardization and validation.

THREAT CATEGORY: VIRAL AGENTS

The countermeasures, technical barriers, and accomplishments in the biological threat category of viral agents are outlined below.

Countermeasures:

- Vaccines for immunity against viral threat agents
- Antibodies and antivirals for treatment of viral disease
- Devices and technologies for diagnosis of viral disease

Technical Barriers:

- Appropriate animal model systems for investigation of viral threats and countermeasures
- Capability to produce GMP pilot lots of vaccine candidates
- Inability to perform human clinical trials to prove efficacy of vaccines
- Production of multivalent vaccines against heterologous viral agents
- Difficulty in optimizing and comparing different expression vectors for recombinant products (vaccines and antibodies)
- Immune enhancement of disease
- Rapid virus identification technology

- Defining surrogate markers of protection

Accomplishments:

Encephalitis Viruses

- Met Milestone 0 pre-clinical technical issues (exit criteria) for a new Venezuelan equine encephalomyelitis (VEE) 1A/B/C vaccine by demonstrating that: the duration of immunity in rodents is greater than one year; the rate of induction of immunity in rodents is one week; there is no neurovirulence in rodents; and there is no reversion to wild type virus in rodents.
- Established methods for production of GMP pilot lot of new VEE vaccine.
- Developed full-length clone of western equine encephalomyelitis (WEE); generated an attenuated WEE vaccine candidate and initiated assessment in rodents.
- Cloned structural genes of eastern equine encephalomyelitis (EEE), and IE and III of VEE and expressed proteins for use as immunogens and diagnostic reagents.

Variola, the Causative Agent of Smallpox

- Identified three drugs as candidates for smallpox therapy.
- Transferred PCR-based diagnostic techniques from the Centers for Disease Control and Prevention (CDC) to the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).
- Expressed vaccinia virus proteins for surrogate marker studies.
- Selected and characterized human monoclonal antibodies which neutralize vaccinia virus.
- Developed aerosol model of monkeypox virus in non-human primates.
- Evaluated efficacy of existing smallpox vaccine against aerosol exposure of monkeypox virus in non-human primates.

Filoviruses

- Developed models for filovirus infections in guinea pigs and mice.
- Assessed the value of passive therapy in filovirus infection, including evaluating the Russian equine immunoglobulin product for the World Health Organization.
- Pathogenesis studies identified the mononuclear phagocyte system as a primary target of Ebola utilizing in-situ hybridization and immunohistochemical staining, and suggested cytokines as a critical factor in pathogenesis.
- Cloned genes and expressed the individual proteins of both Marburg and Ebola viruses in several potential vaccine vehicles.
- Performed initial evaluations of potential vaccine strategies in rodent models.

CONFIRMATORY ASSAYS FOR BIOLOGICAL WARFARE THREAT AGENTS

The accomplishments in the confirmatory assays for biological warfare threat agents are outlined below. The objective of this effort is to develop the capability to confirm in biological samples the initial field diagnosis of a biological warfare threat agent.

- Demonstrated ultrasensitive electrochemiluminescence (ECL) detection of 0.01–100 ng *Yersinia pestis* F1 antigen per milliliter of sera.
- Demonstrated probe-based/microplate assay for field detection of amplified polymerase chain reaction (PCR) products.
- Demonstrated the next generation of probe-based amplified nucleic acid detection using technology compatible with real time quantitative PCR.
- Evaluated methods for rapid specimen preparation for nucleic acid detection methods.
- Improved diagnostic reagents for immunological diagnosis of biological warfare threat agents by doing the following: used automated high-pressure liquid immunoaffinity chromatography to efficiently purify diagnostically-relevant antigens; demonstrated the feasibility of using hyperimmunized laying hens as a source for producing immunoaffinity-purified antibodies; successfully transitioned chicken anti-VEE virus antibody for use in the chromatographic immunoassay format; and, produced immunoreagents and provided them to a commercial contractor for the development and delivery of VEE virus detection chromatographic immunoassays.
- Developed diagnostic methods for the identification and characterization of variola viruses by doing the following: sequenced 4 genes of representative orthopoxviruses; identified and evaluated primers for 7 gene targets; demonstrated "taqman" PCR technology for differentiating between closely related orthopoxviruses; demonstrated large fragment PCR amplification of orthopoxviruses; and, demonstrated the utility of RFLP analysis of orthopoxviruses.
- Demonstrated sensitive nucleic acid based detection assay for *Brucella* species.
- Demonstrated sensitive multiplex nucleic acid based detection assay for *Bacillus anthracis* (the causative organism of anthrax).
- Demonstrated sensitive multiplex nucleic acid based detection assay for *Clostridium botulinum* A/B/E/F.

D.2.3 Advanced Development Accomplishments

- Conducted pre-clinical testing of improved anthrax vaccine for Milestone I transition.
- Investigated safety and efficacy of vaccine candidates for brucellosis and plague in animal models.
- Evaluated Venezuelan equine encephalitis infectious clone vaccine candidate in animal models and prepared data package for Milestone I transition.
- Demonstrated efficacy of subunit vaccine candidates for ricin toxin using *in vivo* models and identified potential surrogate markers of protective immunity.
- Conducted pre-clinical testing of the SEB toxoid vaccine candidate and evaluated second generation vaccine candidates against lethal effects from the toxin.
- Evaluated pharmacological prophylaxis and developed recombinant vaccine candidate expression system, and a GMP level product against botulinum toxins.
- Evaluated candidate systems for sensitive and specific confirmatory diagnosis of viral and bacterial biological warfare (BW) agents in clinical samples.

D.2.4 Joint Vaccine Acquisition Program Accomplishments

The development of vaccines under this program involves studies which demonstrate product safety and efficacy and which are required for product licensure by the FDA. The Joint Vaccine Acquisition Program is managed by the Joint Program Office for Biological Defense. During FY96, the following actions were accomplished:

- Initiated data collection and collation from laboratory studies and manufacturing records to support an FDA license amendment for the anthrax vaccine for a reduced immunization schedule (number of doses for protection).
- Initiated data collection and collation from laboratory studies and manufacturing records to support an FDA license application for botulinum pentavalent vaccine.
- Began clinical evaluations of volunteer and laboratory workers to determine the effects of multiple immunizations with biological defense (BD) vaccines under the special immunization program (long term immunization studies).
- Prepared product license application and establishment license application for tularemia vaccine and submitted both to the FDA.
- Completed independent government cost estimate for BD vaccine prime systems contract.
- Released a request for proposal seeking a comprehensive integrated approach to developing, licensing, producing, testing, and storing BD vaccines (Prime Systems Contract).

D.3 MEDICAL NUCLEAR (RADIOLOGICAL) DEFENSE RESEARCH AND ACQUISITION PROGRAM

D.3.1 Fielded Products

Advances in medical R&D significantly impact the warfighting mission by sustaining unit effectiveness through conserving the fighting strength of our service members. The individual service member whose performance is decremented by disease symptoms is significantly more likely to become a traumatic casualty. In this era of small, but highly lethal forces, loss of only a few team members can dramatically diminish a unit's capability. Medical R&D products (materiel and non-materiel solutions) provide the foundation that ensures the fielding of a flexible, sustainable, modernized force across the spectrum of conflict and in the full breadth and depth of the battlefield. Overcoming medical threats and extending human performance has provided a significant increase in military effectiveness in the past and presents the potential for future enhancement on military operational effectiveness. Some of the fielded materiel and non-materiel solutions by medical radiological defense R&D are:

- *Advances in the Treatment of Radiologic Injuries*, a medical research symposium publication, Permagon Press, Elsivier Science, Ltd.
- North Atlantic Treaty Organization (NATO) Handbook AMedP-6, *Medical Aspects of Nuclear, Biological, and Chemical (NBC) Defensive Operations*
- Medical Effects of Nuclear Weapons Course--Training for approximately 760 Medical Department personnel in FY96.
- Advanced treatment modalities for bone marrow injury, such as the cytokines, which were available for the Gulf War
- New generation antiemetics effective for prevention of early debilitating symptoms of moderate radiation injuries (Now being inserted into NATO doctrine)

D.3.2 Nuclear Defense Research and Development Accomplishments

The nuclear (or radiological) defense research and development technical barriers and accomplishments during FY96 are grouped in the following threat categories:

- Prompt radiation from nuclear weapons,
- Protracted low level radiation from fallout and other sources
- Combined effects of radiation and other factors

"*Prompt radiation*" refers to the high level radiation released by a nuclear weapon detonation in the first sixty seconds after the explosion. Significant injury occurs within seconds of exposure. "*Protracted low level radiation*" refers to radiation from nuclear fallout, radiological dissemination devices, and other sources which contaminate an area with radioactive particles. The exposure time required to cause casualties in this environment is much longer than the

instantaneous exposure of prompt radiation. The "*combined effects*" environment significantly augments the casualty rate by amplifying the subclinical effects of traditional trauma, burns, wounds, and infection. Due to the likelihood of an enemy's simultaneous use of nuclear dissemination weapons and chemical/biological agents, combined injury effects now also must include the previously unresearched interactions of low level radiation and chemical-biological weapons.

THREAT CATEGORY: PROMPT RADIATION

The countermeasures, technical barriers, and accomplishments in the threat area of prompt radiation are outlined below.

Countermeasures:

- Advanced medical treatment strategies for radiation injuries
- Drugs designed to increase resistance of soldiers to radiation and protect the soldier against radiation injury without compromising performance
- Drugs designed to prevent the onset of radiation-induced performance decrements such as fatigue, nausea, vomiting
- Assessment of radiation injury by biological dosimetry techniques

Technical Barriers:

- Known drugs that provide some radiation protective effects have serious performance-degrading side effects at drug doses required for operational requirements
- Mechanisms of action of several known treatment and radioprotective drug strategies are not well understood
- Drug delivery system which allows extended bioavailability is not available for radioprotectants

Status:

- Research in collaboration with pharmaceutical companies using large and small animal models is on-going
- Research using cellular systems and rodents has begun to investigate strategies to mitigate against late effects (*e.g.*, cancer) of radiation
- Research using cellular systems and rodents has begun to investigate strategies to mitigate infection in irradiated animals
- Combination of drugs administered at non-toxic levels which provides protection has been identified
- Biological dosimetry techniques based on cytogenetic techniques are being validated and developed for fielding
- Greater emphasis is being provided on molecular and cellular biology strategies to elucidate mechanisms of radiation damage and protection

Accomplishments:

- Devised and tested prophylactic/therapeutic protocols to show efficacy in reducing the duration of neutropenia and thrombocytopenia.
- Demonstrated that lethal consequences of radiation can be averted with the therapeutic use of cytokines.
- Established new generation blocking agents which can reverse endotoxin shock.
- Devised drug combinations that can provide a margin of safety against ionizing radiation lethality without compromising performance.
- Demonstrated dose assessment techniques based on cytogenetic techniques
- Developed molecular and cellular model systems to validate new approaches to enhance resistance to ionizing radiation.
- Devised therapeutic protocols which combine selected immunomodulators and antibiotics and show efficacy in reducing lethality from infection in irradiated animals.

THREAT CATEGORY: PROTRACTED LOW LEVEL RADIATION

The countermeasures, technical barriers, and accomplishments in the threat area of protracted low level radiation from nuclear fallout, radiological explosive devices, *etc.*, are outlined below.

Countermeasures:

- Advanced medical treatment strategies for protracted radiation injuries from both external and internal sources of radioactivity
- Drugs designed to protect personnel from the early and late effects of ionizing radiation without compromising performance
- Improved techniques to detect and remove internal sources of radioactivity
- Improved drug delivery system to provide protection during the entire period of radiation exposure

Technical Barriers:

- Availability of suitable radiation sources to study the effects of chronic exposure at relevant dose levels
- Difficulty in manipulating cellular repair mechanisms
- Toxicity of chelating agents used to remove sources of radioactivity
- Brief periods in which traditional radioprotective drugs are active
- Toxicity of radioprotective drugs used over protracted periods of time
- Lack of sustained drug delivery system for radioprotectants
- Microbial resistance to antibiotics

Status:

- New facility to permit protracted radiation exposure experiments is being planned to model current and future threat scenarios
- New biological models for internal and external cellular and whole-body chronic exposure studies are being developed
- New programs have been instituted for the study of molecular biology approaches to study gene radiation damage and repair mechanisms
- Novel drug delivery systems (*e.g.*, transdermal patches) are being evaluated for efficacy in providing protection in chronic radiation environments

Accomplishments:

- Established contracts to study chronic human exposures with scientists within the former Soviet Union
- Demonstrated that synaptic potentials in central nervous system neurons show anomalous dose-rate dependence
- Confirmed that low-dose-rate neutrons have an increased rate of oncogenic transformation for certain specific cell lines

THREAT CATEGORY: COMBINED EFFECTS.

The countermeasures, technical barriers, and accomplishments in the threat area of combined effects of nuclear radiation and trauma, burns, and infection are outlined below.

Countermeasures:

- Radiotherapeutic agents designed to decrease morbidity and mortality from multi-organ system failure due to the combined effects of radiation, trauma, burns, and infection
- Radioprotective drugs designed to harden the soldier against the effects of radiation, trauma, burns, and infection
- Combined therapeutic agents designed to decrease morbidity and mortality from and to enhance innate immune responses
- Computer models for predicting casualties following combined exposure to low levels of ionizing radiation and BW/CW agent aerosols

Technical Barriers:

- Availability of reliable animal models to predict effects in humans
- Antimicrobial resistance to current antimicrobial therapeutic agents
- Differences sensitivities of biological systems at all levels to neutrons and gamma rays
- Mechanism of action of cell-growth factors is not well understood
- Sensitivity of bone marrow progenitor cells to low doses of ionizing radiation

Status:

- Research in collaboration with pharmaceutical companies using small and large animal models continues
- Evaluations of radioprotective and radiotherapeutic agents on-going in mixed-field irradiated animal models
- New antimicrobial products under evaluation for the treatment of gram-positive and gram-negative bacterial sepsis in irradiated rodents.
- New immunomodulators evaluated for enhancing innate immune responses against infections.
- Molecular biology techniques utilized to understand the effects of radiation, trauma, and combined effects
- Molecular biology techniques utilized to understand the beneficial effects of cell growth factors, immunomodulators, and antimicrobial agents

Accomplishments:

- Demonstrated that selected radioprotective drugs reduce mortality from combined effects in small animal models
- Demonstrated that selected antimicrobial agents promote survival from infection when given orally to mixed-field irradiated small animal models
- Demonstrated that combined modality therapy including topical/systemic antimicrobial agents, immunomodulators, and radioprotective drugs increase survival from combined effects
- Calculated effectiveness of low yield nuclear weapon for neutralization of BW stockpile using Armed Forces Radiobiology Research Institute (AFRRI) radiation-kill curves for [anthrax] spores of *Bacillus anthracis* (Sterne strain) and spores of three other *Bacillus* species
- Demonstrated that sublethal irradiation significantly decreased survival of mice challenged with [anthrax] spores of *B. anthracis* (Sterne strain)
- Developed first generation model for the interaction of radiation with a biological agent

D.3.3 Predevelopment Products

Technical developments in predevelopment products for medical radiological defense include the following:

- Medical Effects of Nuclear Weapons CD-ROM interactive training program for military health care personnel
- Pre-Transition Information Paper: *Radioprotection by a Combination of Iloprost/Misoprostol/3D-MPL/WR-3689*
- Automated biodosimetry capability based on lymphocyte dicentric analysis.

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